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VEGF Receptor Tyrosine Kinases

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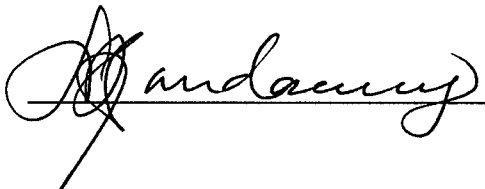
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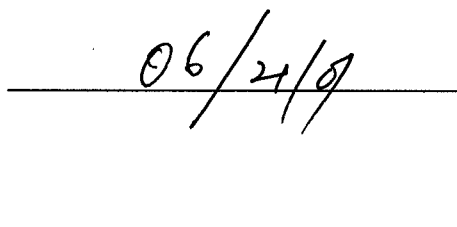
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13. ABSTRACT (Maximum 200 Words) Angiogenesis plays a fundamental role in solid tumor growth and metastasis. The vasculature is for the most part quiescent in the adult, so it follows that anti-angiogenic therapies are an attractive method of targeting tumorigenesis. Thus, the purpose of this study was to investigate the basic mechanisms behind vascular development with the goal of discovering novel proteins involved in angiogenic processes. VEGF (Vascular Endothelial Growth Factor) is a growth factor that has been proven to promote angiogenesis in solid tumors. We undertook two screens: the first to identify the genes transcriptionally regulated by VEGF stimulation of endothelial cells, and the second to identify proteins which participate in the signal transduction pathways downstream of VEGF receptors. Our work focused on SOCS2 (Suppressor of Cytokine Signaling, an SH2-containing cytoplasmic protein. SOCS2 was pulled out of yeast two-hybrid screen for proteins that interact with VEGFR1. SOCS2 was present in the endothelium and associated specifically with stimulated VEGF receptors in a GST-pulldown system, suggesting a role in the regulation of angiogenesis. This project both informs the understanding of angiogenesis and has the potential benefit of identifying a novel, specific target for anti-angiogenic therapy.				
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## Introduction

Angiogenesis, or blood vessel growth, plays a fundamental role in solid tumor growth and metastasis. The vasculature is for the most part quiescent in the adult, so it follows that anti-angiogenic therapies are an attractive method of targeting tumorigenesis. Thus, the purpose of this study was to investigate the basic mechanisms behind vascular development with the goal of discovering novel proteins involved in angiogenic processes. Vascular Endothelial Growth Factor (VEGF) is a growth factor that has been proven to promote angiogenesis in solid tumors. We undertook a two screens: the first to identify the genes transcriptionally regulated by VEGF stimulation of endothelial cells, and the second to identify proteins which participate in the signal transduction pathways downstream of VEGF receptors. This project both informs the understanding of angiogenesis and has the potential benefit of identifying novel, specific targets for anti-angiogenic therapy.

## Body

### Identification of Genes Transcriptionally Regulated by VEGF Receptor Tyrosine Kinases (RTKs)

This project was covered in the 1999 Annual Report.

Specific Aim 1 – Establish an *in vitro* system in which to study VEGF RTKs

Specific Aim 2 – Clone genes differentially expressed in the endothelium as a result of VEGF RTK activation.

We performed a cDNA Representational Difference Analysis (RDA) screen for genes expressed differentially between unstimulated and VEGF-stimulated endothelial cells. Cloned potential genes were further screened by Northern Blotting. Of the potential genes, we focused on CD9, a cell surface protein that seemed to play a role in migration in other systems. Although antibodies to CD9 were able to retard endothelial cell migration towards chemotactic stimuli *in vitro*, this effect was not found to be specific to VEGF stimulation. Technical difficulties hampered the characterization of endothelial cell surface expression and its potential regulation by VEGF stimulation. Characterization of CD9 and its role in endothelial cell migration in angiogenesis was deemed by the principal investigator's graduate advisory committee to be too far afield from the laboratory's expertise, and was terminated in favor of another project. Thus Specific Aim 3 – Characterize the function of genes differentially expressed in the endothelium after stimulation of VEGF RTKs was "overtaken by events", while the first two specific aims were performed as planned.

As also explained in the 1999 Annual Report, an event occurred which directed the course of my research.

- Dr. Kevin Peters, my research mentor, left Duke University Medical Center for a position in the private sector.
- My thesis work was completed in the laboratory of Dr. Chris Kontos, whose background is in cellular signaling.

Due to the change of laboratories, it was agreed upon that my project should focus upon the proteins involved in signaling downstream of the VEGF receptors in order to take advantage of the expertise of my surroundings.

### Identification of Cell Signaling Proteins Which Associate With the VEGF Receptor

The second project follows the same ideas as the initial project focusing on transcriptionally regulation by VEGF. The two projects both involve identifying the basic mechanisms of a pathway known to stimulate angiogenesis in solid tumor growth. The only difference is the part of the pathway being investigated: instead of genes transcriptionally regulated by VEGF stimulation of VEGF receptors, we concentrated on the cytoplasmic proteins directly interacting with the stimulated VEGF receptors. Thus, the specific aims of the signaling project were as follows.

Specific Aim 1 – Clone genes which potentially interact with the activated VEGF receptors.

Specific Aim 2 – Characterize interaction of VEGF receptors and interactors *in vitro*.

Specific Aim 3 – Determine effect of protein interactors on the growth and maintenance of the vascular network

Specific Aim 1 – Clone genes which potentially interact with the activated VEGF receptors.

The VEGF Receptor1 (VEGFR1, Flt-1) kinase domain was used as bait in a yeast-two hybrid screen of a human fetal heart library (in collaboration with M. Blonar, Bristol-Myers Squibb). This screen demonstrated a novel association between Flt-1 and SOCS2, a SH2-domain containing cytoplasmic protein. (2000 Annual Report)

The SOCS family, so named for their function as “Suppressors Of Cytokine Signaling” display a homology via their C-terminal “SOCS box”. The SOCS box has a characteristic N-terminal BC box and C-terminal L/P rich sequence, and its function has been under debate: it is either a protein stabilizer or a targeting protein for protein degradation. Several members of this family have been shown to function as negative regulators of signal transduction. They affect JAK/STAT signaling via the SH2 domain and an N terminal kinase inhibitory region. VEGF stimulation of its cognate receptors has been demonstrated to activate Stat signaling pathways. Thus, our hypothesis is that SOCS2 modulates VEGF-induced angiogenesis by binding to the RTK via its SH2 domain.

#### Specific Aim 2 – Characterize interaction of VEGF receptors and interactors *in vitro*.

1. The association of VEGFR1 and VEGFR2 (Vascular Endothelial Growth Factor 2, Flk-1) with SOCS2 has been demonstrated in both the yeast two-hybrid system and a GST-pulldown system.
2. This association is dependent upon the presence of both a functional kinase domain on the receptor and an intact SH2 domain in SOCS2.
3. Expression studies have detected SOCS2 mRNA in highly vascularized tissues and SOCS2 protein in the endothelium of the placenta, adding weight to the hypothesis that the *in vitro* association between SOCS2 and VEGF receptors occurs *in vivo*. (2000 Annual Report)

These findings were presented as a poster entitled “Role of SOCS2 in VEGF and Tie-mediated angiogenesis”, Era of Hope Meeting, AMRMC Breast Cancer Research Program, June 2000. The results received positive feedback from other investigators present.

#### Specific Aim 3 – Determine effect of protein interactors on the growth and maintenance of the vascular network

3A. Determine how VEGF stimulation regulates SOCS2 association with VEGF receptors in endothelial cells. Experiments were performed to determine whether SOCS2 association was affected by VEGF stimulation of endothelial cells. Specifically, VEGF-165 was used to stimulate endothelial cells in culture: HUVEC (Human Umbilical Vein Endothelial Cells, which express both VEGF receptors) or BAEC (Bovine Aortic Endothelial Cells) stably transfected with VEGFR1, VEGFR2, or untransfected controls. SOCS2 was expressed by transient lipofectamine-mediated transfection and visualized by an anti-Flag antibody. Initial results suggested a time-dependent association of SOCS2 with VEGFR1 post-VEGF stimulation. Unfortunately, these results could not be conclusively confirmed within the parameters set forth. These experiments are technically very difficult, requiring a combination of reliable antibodies, good transfection efficiency, sufficient receptor expression, proper timing, and sufficient washing to decrease background yet not completely interfere with protein-protein associations. Thus, our failure to conclusively demonstrate VEGF regulation of the association between SOCS2 and the VEGF receptors does not rule out its occurrence in nature.

3B. Determine function of SOCS2 interaction with VEGFR in angiogenesis.

We made an adenovirus that caused overexpression of SOCS2 in infected cells to facilitate functional experiments. We were unable to create an adenovirus overexpressing a mutant SOCS2 containing a point mutant in the consensus SH2 binding domain, most likely attributable to cellular toxicity due to viral protein overexpression. SOCS2 overexpression was used in a tritiated thymidine cell proliferation assay to determine its effect on VEGF-stimulated endothelial cell proliferation. At timepoints ranging from 3 to 96 hours SOCS2 overexpression did not significantly alter VEGF-stimulated endothelial cell proliferation. The adenovirus was also used to infect rat aortic rings grown in culture in an assay of endothelial sprouting. Treatment with the SOCS2 adenovirus did not completely abolish endothelial sprouting, as does an adenovirus expressing the phosphatase SHPTP2. The rat aortic ring assay is “all-or-nothing” – dramatic results are excellent and believable but partial suppression of endothelial sprouting is difficult to quantitate. Thus, this line of

experimentation was put aside. SOCS2 may have a function in endothelial cell migration or in the more amorphous "vascular maintenance" that remain to be seen.

These results have been written up and submitted to the Journal of Biological Chemistry (See appendix).

## **Key Research Accomplishments:**

Completion of cDNA RDA screen for genes transcriptionally regulated by VEGF stimulation

Completion of yeast-two hybrid screen for protein interactors with VEGFR1 receptor.

Characterization of the expression and association of SOCS2, a cytoplasmic SH2 domain containing signaling protein, with VEGFR1 and VEGFR2.

## **Reportable Outcomes:**

Successful defense of Ph.D. degree in Cell Biology in December 2000.

Wong AL, Rao PS, Kontos CD, Peters KG: Novel Association Between VEGFR1 and VEGFR2 and SOCS2, a cytoplasmic SH2 containing protein. Submitted.

Published Abstract and Oral Presentation, "Novel Association between SOCS2 and VEGFR1 and VEGFR2: Implications in Vascular Signal Transduction and Angiogenesis."  
**American Heart Association 73<sup>rd</sup> Annual Scientific Sessions**, November 2000.

Poster Presentation, "Role of SOCS2 in VEGF and Tie-mediated angiogenesis",  
**Era of Hope Meeting, AMRMC Breast Cancer Research Program**, June 2000.

Poster Presentation, "Novel Association between Flt-1, Flk-1, Tie2 and SOCS2 in the yeast two-hybrid system", **American Society for Cell Biology Annual Meeting**, December 1999

Appointment as a Postdoctoral Research Scientist in the Laboratory of Dr. Frank Costantini in the Dept. of Genetics and Development at Columbia University Physicians and Surgeons, New York. I will be working on branching morphogenesis in the kidney, and the regulatory role of the RTK RET in morphogenesis.

## Conclusions:

### Functional Significance of Transcriptional Regulation by VEGF Receptor Tyrosine Kinases

In our first screen, CD9 was identified by an cDNA RDA screen as a gene transcriptionally regulated by VEGF stimulation of ECRF cells. Its expression increases two fold over 16 hours post stimulation by Northern blotting. Early FACS results demonstrating a rapid upregulation of CD9 at the cell surface in response to VEGF stimulation proved difficult to duplicate. The addition of a CD9 blocking antibody (Syb-1) rendered VEGF-mediated HUVEC migration 50% less efficient. Thus, we believe that the Syb-1 effect on cell migration is an indirect result of binding CD9 on the cell surface and is not specific to VEGF-mediated cell migration.

The tetraspanin field is hampered by the lack of good antibodies, making it difficult to clearly determine protein-protein interactions. While Syb-1 does indeed block migration, its epitope has never been mapped, rendering structure-function speculation difficult. Attempts to investigate differential protein association upon VEGF stimulation were also stymied by the lack of a clean antibody to perform coimmunoprecipitation experiments. Another difficulty lay in pursuing a line of investigation more focused on integrins and cell motility when the lab's focus was RTK signaling. Another group published very similar results demonstrating the role of CD9 in migration of endothelial cells: CD9 colocalized with integrin  $\beta 1$  and  $\beta 3$  on the cell surface, and an anti-CD9 antibody blocked cell migration towards fibronectin and vitronectin (1).

The failure of this screen to identify genes transcriptionally regulated by VEGF stimulation of ECRF cells could be attributable to many factors. For example, we may have chosen the wrong timepoint post-VEGF expression. Alternatively, genes relevant to VEGF signaling might be activated in a paracrine fashion undetectable by our single cell type system. Technical difficulties might lie in the cloning strategy. For instance, insufficient subtraction might not have removed all non-regulated transcripts. While this approach to subtractive cloning was very popular at the time, there have been few recent published accounts, suggesting problems encountered by other users. While a two-fold change in mRNA expression may well be relevant, a larger change in transcription levels might be reassuring before embarking upon experimentation. Finally, the fact that the same transcript did not appear multiple times when sorting through the positive clones should have been a warning sign that the cloning strategy was imperfect.

## Identification of Cell Signaling Proteins that Associate with the VEGF Receptor

Our results demonstrate a novel association between VEGF receptors 1 and 2 and the SH2 domain containing SOCS2 protein. Both VEGFR1 and the closely related VEGFR2 interacted with SOCS2 in a kinase-dependent manner in the two-hybrid system. Experiments in a GST-pulldown system confirmed the association between the VEGFRs and SOCS2 and demonstrated a dependence upon the presence of an intact SOCS2 SH2 domain. Finally, the demonstration of SOCS2 protein expression in endothelial cell lines and in the endothelium *in vivo* further supports the possibility of an *in vivo* role for the association between SOCS2 and the VEGFRs.

SOCS family members share a role in the negative regulation of signal transduction (2). SOCS proteins are upregulated as a result of cytokine transcription, and then negatively regulate cytokine signaling via disruption of the Jak/Stat pathway creating a classical negative feedback loop. Activation of the STAT pathway has been demonstrated in VEGF signaling (3) (4). STAT activation by VEGF stimulation suggests that SOCS genes should be upregulated, which, when taken together with our findings, suggests a potential negative feedback regulation of VEGF signaling by SOCS proteins.

Like other SOCS family members, SOCS2 expression is tightly regulated in many cell types, with low resting levels of expression (2). The nature of SOCS2 induction in the endothelium could be an important determinant of VEGFR-SOCS2 function. A negative feedback model suggests VEGF-induced STAT activation as a candidate for SOCS2 regulation. Other angiogenic growth factors such as the angiopoietins, FGF, or prolactin might also affect SOCS transcription. Similarly, cytokines could impact VEGF signaling by stimulating STAT and upregulating SOCS transcription. The identification of the pathways that regulate SOCS2 protein production in the endothelium will be an important addition to the understanding of its role in angiogenesis.

SOCS family members employ unique mechanisms for regulating signal transduction. The requirement for an intact SH2 domain in SOCS2 to bind the VEGF receptors suggests the SOCS2 association is with a specific phosphotyrosine. Supporting this possibility, preliminary experiments have shown that SOCS2 has no effect the autophosphorylation of recombinant VEGFR1. Future experiments will determine the specific binding site for SOCS2 on VEGFR1 and VEGFR2, and clarify the function of SOCS2 association with the VEGFRs.

Once expressed, SOCS2 can perform different functions depending on its cellular concentration. Low levels of transfected SOCS2 protein suppress GH induced transcription of Stat5, whereas higher levels actually cause superinduction of transcription (5). SOCS2 has only a partial inhibitory effect on other types of cytokine signaling, independent of its concentration (2). Coexpression of SOCS2 can modulate SOCS1 suppression of Stat5 transcription at higher concentrations (5, 6). Thus, it will be important to determine what other SOCS proteins are involved in VEGF signaling and how they interact to affect angiogenesis.

While the association of SOCS2 with both VEGFR1 and VEGFR2 suggests that the SOCS protein acts as a general modulator of the VEGF response, stimulation with PlGF vs. VEGF or receptor-specific mutants of VEGF will reveal the specificity of SOCS2 regulation of the VEGF receptors *in vivo*.

Thus, this research has revealed a novel association between the activated VEGF receptors and the SOCS family of negative regulatory proteins. The relationship between the activated form of an angiogenic growth factor receptor and SOCS has leads to intriguing models of SOCS protein regulation of VEGF-induced angiogenesis. While there is still much work to be done to characterize the *in vivo* expression of SOCS2 and its possible regulation of VEGF induced angiogenesis, the identification of their interaction has been an important step in the elucidation of the mechanisms governing angiogenesis.

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Novel Association Between SOCS2 and VEGFR1 and VEGFR2  
**Novel association between SOCS2 and VEGFR1 & VEGFR2**

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**Abstract**

VEGF stimulation of its receptors, VEGFR1 and VEGFR2, plays an essential role in angiogenesis. In order to find new members of VEGF-mediated signaling pathways, a yeast two-hybrid screen of a human fetal heart library was performed using the VEGFR1 kinase domain as bait. One of the proteins identified in this screen was SOCS2, a member of a family of SH2 domain-containing cytoplasmic signaling proteins that act as negative regulators of cell signaling. SOCS2 mRNA was broadly expressed in adult human tissues, and SOCS2 protein could be detected in endothelial cell lines and in the endothelium of human placental tissue. Further bait testing in the two-hybrid system showed that SOCS2 interacted with both VEGFR1 and VEGFR2 in a kinase-dependent manner. In a GST pulldown assay, the association between the VEGFR1 and VEGFR2 kinase domains and SOCS2 was dependent upon the presence of both a functional kinase domain and an intact SOCS2 SH2 domain. These studies demonstrate that SOCS2 is expressed in endothelial cells and that it associates with both VEGFR1 and VEGFR2, suggesting a potential role for SOCS2 in the regulation of VEGF mediated signaling. Future studies will determine the importance of SOCS2, and perhaps other members of the SOCS family, in the regulation of VEGF-induced signaling and angiogenesis.

## ***Introduction***

Vascular Endothelial Growth Factor (VEGF) is an important mediator of vascular growth and development (1,2). VEGF stimulation leads to the activation of its cognate receptor tyrosine kinases (RTKs), VEGFR1 and VEGFR2, which are expressed predominantly in the vascular endothelium. Ligand binding to the VEGFRs leads to dimerization and autophosphorylation of specific cytoplasmic tyrosine residues. These phosphotyrosine residues then serve as anchor points for signaling proteins that activate downstream signal transduction leading to changes in cellular behavior (3).

During development of the embryonic vasculature, the two VEGF receptors have distinct, nonoverlapping functions, as demonstrated by knockout experiments. Disruption of VEGFR1 leads to embryonic lethality characterized by severe disorganization of the vascular system and an increased number of endothelial cells (4,5). VEGFR2 knockout mice are also embryonic lethal, characterized by a defect in endothelial and hematopoietic cell maturation resulting in a complete lack of endothelial cells (6). The distinct phenotypes of the VEGFR knockout mice support the existence of distinct signal transduction pathways downstream of VEGF receptors.

*In vitro* experiments have demonstrated shared and unique signaling partners and cellular responses downstream of the two VEGF receptors. For example, p85 binding to the activated VEGFR2 results in the activation of PI3-Kinase leading to cellular migration, proliferation, and

Novel Association Between SOCS2 and VEGFR1 and VEGFR2 cell survival (7-9). In contrast, the demonstration of p85 interaction with VEGFR1 in the yeast two-hybrid system, has not been borne out *in vivo* (10,11). The activation of the MAP kinase pathway is common to both VEGF receptors, although the proliferative effect of VEGFR2 may be more potent than VEGFR1(12,13). PLC $\gamma$  associates with both receptors *in vitro*, but downstream effects on vascular permeability and migration have only been demonstrated with VEGFR2 (14). Thus, signaling molecules that associate with VEGF RTKs play important roles in the activation of second messenger pathways that contribute to the VEGF induced cellular responses; differences in signaling may, in part, explain the differences in VEGF receptor function.

In order to further investigate signal transduction pathways downstream of VEGFR1, a yeast two-hybrid screen of a human fetal heart library was performed using the VEGFR1 kinase domain as bait. One of the proteins isolated in this screen was SOCS2, a member of a family of proteins that shares the ability to negatively regulate cytokine signaling (15). SOCS2 mRNA was broadly expressed; SOCS2 protein was detectable in endothelial cell lines by Western blotting and in the placental endothelium by immunohistochemistry, suggesting the functional significance of its interaction with VEGF receptors. Further experiments in the two-hybrid system and in a GST pulldown system demonstrated that the SOCS2 interaction with VEGFR1 and VEGFR2 was dependent on the presence of an intact VEGFR kinase domain and an intact SOCS2 SH2 domain. Considering the role that SOCS proteins play in cytokine signaling, these

Novel Association Between SOCS2 and VEGFR1 and VEGFR2 results suggest a novel role for SOCS2 in the modulation of VEGF-mediated signal transduction and angiogenesis.

### ***Materials and Methods***

**Yeast two-hybrid screen & bait testing.** Human fetal heart cDNA library construction, generation and testing of bait plasmids, yeast two-hybrid screening, and the categorization and specificity testing of interactors were performed as described previously (16,17). Kinase-inactive mutants of each receptor were generated using site-directed mutagenesis to mutate the ATP binding site from lysine to arginine (designated KR). All yeast transformations were performed with the Yeastmaker yeast transformation kit (Clontech), and transformants selected by growth on the appropriate dropout medias (Bio 101). Yeast colonies that demonstrated galactose-dependent activation of both the lacZ and LEU2 reporters were considered true positive interactors and were evaluated further.

cDNA from specific interactors was sequenced and evaluated by the BLAST search tool at the NCBI internet site ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). A full-length transcript of the SH2 domain containing clone B4.2 was obtained by RACE PCR from "Marathon-ready" human placenta cDNA (Clontech).

Novel Association Between SOCS2 and VEGFR1 and VEGFR2

**Northern blots.** To assess mRNA expression, a human multiple tissue northern blot (Clontech) was probed with a 32P-labelled (Amersham) random-primed (Prime-it II kit, Stratagene) PstI fragment of SOCS2.

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**Recombinant SOCS2 protein production.** SOCS2 cDNA was subcloned into pET15b, a His-tagged bacterial expression vector (Novagen), sequenced, and transformed into AD494 (DE3) (Novagen), a *trx*B- expression host which allows disulfide bond formation in *E. coli* cytoplasm. Bacteria were grown to OD<sub>600</sub>=0.5 in the presence of 50 mg/ml ampicillin and 15 mg/ml kanamycin (Sigma), induced for 3 hr with 1 mM IPTG (Sigma) and 0.5 mM PMSF, and collected by centrifugation. Proteins were extracted with Bugbuster Protein Extraction Reagent (Novagen) and lysozyme (Sigma) and centrifuged at 16,000g for 15 min. The pellet was solubilized in 6 M guanadine-HCl, centrifuged at 10,000 g for 20 m, and soluble HisSOCS2 was bound to pre-equilibrated NiNTA-agarose (Qiagen) for 1 hr at room temperature on a nutator. Denatured protein was eluted directly with imidazole. For renatured His-SOCS2 protein, Ni-NTA bound HisSOCS2 was renatured over a step gradient from 2M to 0M GuHCl (in 1mM reduced glutathione 0.3 mM oxidized glutathione, 500 mM NaCl 20 mM Tris pH 7.5, 20 mM imidazole, 10 mM βME containing 1 mM PMSF, 1 μg/ml pepstatin and leupeptin), then eluted in the same buffer with 125 mM imidazole. Proteins were then dialyzed against PBS to remove the imidazole.

**SOCS2 Antibody production & purification.** Rabbit polyclonal serum against purified denatured His-SOCS2 was produced by Animal Pharm Services, Heraldsburg, CA. Affinity purification of the rabbit polyclonal serum was performed as follows: Renatured His-SOCS2 was

Novel Association Between SOCS2 and VEGFR1 and VEGFR2 coupled overnight to activated CNBr powder (AmershamPharmacia), the remaining active groups were blocked with 0.1 M Tris pH 8 for 2 hr, then washed (PBS, 0.2% Triton, 1 mM EDTA, 1mM NaN<sub>3</sub>, 1 M NaCl) and equilibrated with the same buffer with no NaCl. Serum was mixed 1:1 with PBS, 0.2% Triton, 1 mM EDTA, 1mM NaN<sub>3</sub>, heated for 10 m at 55° with 100 ug/ml PMSF, and insoluble debris was pelleted. The supernatant was passed over the column twice, then the column was then washed first with equilibration buffer and then with PBS without Triton until A280=0. Antibody was eluted with Pierce Immunopure IgG elution buffer (Pierce), immediately neutralized in 1/10 V 1 M Tris pH 8.0, and dialyzed against PBS pH 7.4.

**Cell lines, transfections and tissues.** HEK-293 cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium DMEM media (Life Technologies) 10% FBS (Hyclone, Logan, UT) 100 U penicillin/ml media and 100 µg/ml media streptomycin (Life Technologies). 293 cells were transiently transfected with Lipofectamine Plus reagent (Life Technologies) in Opti-MEM I media (Life Technologies) with 4 µg DNA per 10 cm plate of ~80% confluent cells for 3 hr. HUVEC, primary human umbilical vein endothelial cells (Clonetics), were cultured in EGM with 10% FBS (Clonetics). PAE, immortalized porcine aortic endothelial cells (gift from Tim Quinn, UCSF) were cultured in F-12 media (Life Technologies) with 10% FBS 1% Pen/Strep. sf9 cells were passaged in suspension in Grace's Supplemented Insect Medium (Life Technologies) with 10% FBS and 0.1% Pluronic F-68 (Life Technologies) 100 U penicillin/ml,

Novel Association Between SOCS2 and VEGFR1 and VEGFR2  
0.1 mg streptomycin/ml and 0.25 µg amphotericin B/ml (Sigma). Cells were lysed in triton lysis buffer: 20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1% Triton X100 with 1 mM PMSF, 1 µg/ml pepstatin and leupeptin. Post-partum human placenta was obtained as anonymous discarded tissue from the Duke University Medical Center Cord Blood Facility. Tissue was lysed in modified RIPA buffer: 50 mM Tris pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 µg/ml pepstatin and 1 µg/ml leupeptin.

**Antibodies.** Antibodies were used at the following dilutions or concentrations for Western blotting: GST 1:10,000 (Santa Cruz Biotechnology), MoAb PY99 1:1000 (Santa Cruz Biotechnology), MoAb Flag M2 10 µg/ml TBST (Sigma), PLC-γ 1 µg/ml TBST (Upstate Biotechnology) VEGFR1 1:1000 and VEGFR2 1:1000 (Santa Cruz Biotechnology).

**Immunohistochemistry** Cryosectioned fresh-frozen human placental tissue was acetone-fixed for 10 m @ -20°, blocked for 20m @ rt in 1% normal serum in PBS, incubated in primary Ab for 1 hr at rt with purified polyclonal anti-SOCS2, purified preimmune serum, or anti-CD31 (1:2000, R&D Systems). Staining was performed with the ABC and AP kits from Vector, slides were counterstained with hematoxylin, mounted with Cytoseal 60 (Stephens Scientific) and viewed

Novel Association Between SOCS2 and VEGFR1 and VEGFR2  
with an Olympus IX70 inverted system microscope connected to an Optronics Engineering DEI-  
750 digital camera.

**Association Assays.** Expression and purification of the VEGFR1 and VEGFR2 kinase domains was performed as previously described (16). Briefly, a 5' GST tag was added to cDNAs, which were then cloned into pVL1393 (Pharmlngen) and cotransfected with baculovirus backbone DNA (Pharmlngen) into Sf9 insect cells for expression. Wildtype and kinase-deficient (KR) GST-VEGFR1 and GST-VEGFR2 were purified from Sf9 lysates in Triton-X lysis buffer by overnight incubation with 30  $\mu$ l glutathione-sepharose (18) (AmershamPharmacia). Glutathione-sepharose-bound VEGFR kinase domains were subjected to an *in vitro* kinase reaction for 20 min at room temperature in 100  $\mu$ l of kinase buffer (100 mM NaCl 12 mM MgCl<sub>2</sub>, 1 mM DTT, 20 mM Tris pH 7.5) containing 1 mM ATP.

293 cells were transiently transfected with plasmids encoding pFlag vector, pFlagSOCS2, or pFlag SOCS2RK – which contains a point mutation in the consensus FLVRES motif abrogating SH2 binding (18) (gift of TA Willson and SE Nicholson, The Walter and Eliza Hall Institute of Medical Research and the Cooperative Research Center for Cellular Growth Factors, Parkville, Victoria, Australia (19)).

Whole cell lysates from transfected and untransfected 293 cells were incubated with the bead-bound GST-VEGFR kinase domains for 2 hr at 4°C. The bead-bound VEGFR kinase and

Novel Association Between SOCS2 and VEGFR1 and VEGFR2 associated proteins were then washed 3x in 1 ml cold Triton-X lysis buffer with protease inhibitors and 1 mM sodium orthovanadate, and eluted by boiling into 2x Laemmli sample buffer. Bead-bound VEGFR kinase and its associated proteins were run out alongside of 293 cell lysates as controls on 8-16% Tris-glycine gradient gels (Invitrogen/Novex) and transferred to nitrocellulose (Schleicher & Schuell, Inc.) for Western blotting.

## **Results**

### **SOCS2 interacts with VEGFR1 and VEGFR2 in the yeast two-hybrid system**

In order to discover novel signaling partners for VEGFR1, the entire VEGFR1 intracellular domain was used to screen a library derived from human fetal heart, a site of active angiogenesis. One of the clones identified by this screen was a novel SH2 domain containing protein. The full-length cDNA was cloned by RACE PCR, blast searched, and found to be identical to the published SOCS2 protein. The SOCS family is characterized structurally by a central SH2 domain flanked by an N terminal domain of variable length and a C-terminal SOCS box which associates with elongins B and C (15,20). Functionally, SOCS proteins are induced by cytokine stimulation and play a role in negative feedback regulation of Jak/STAT signaling. Thus, we hypothesized that SOCS2 might function as a negative regulator of VEGFR signaling.

#### Novel Association Between SOCS2 and VEGFR1 and VEGFR2

Since VEGF stimulation leads to activation of signal transduction pathways emanating from two closely related receptors, VEGFR1 and VEGFR2, we used the two-hybrid system to determine whether the SOCS2 might also interact with VEGFR2 (21-23). In fact, SOCS2 interacted with both VEGFR1 and VEGFR2 (Fig. 1). To determine whether SOCS2 preferentially interacted with the tyrosine-phosphorylated, "activated" form of the VEGFRs, we used site directed mutagenesis to create point mutants in the ATP binding sites of the VEGFRs which render the proteins kinase dead (VEGFR1 K861R, VEGFR2 K951R). As shown in figure 1, SOCS2 does not interact with the kinase-dead forms of VEGFR1 or VEGFR2. Thus, in the yeast two-hybrid system, both VEGFR1 and VEGFR2 interact with SOCS2 in a kinase-dependent manner.

#### SOCS2 is expressed in the endothelium

The relevance of the interaction between phosphorylated VEGFR1 & VEGFR2 and SOCS2 in the yeast two-hybrid system is dependent on the presence of SOCS2 in the endothelium. SOCS proteins display characteristic temporal and spatial regulation upon cytokine or growth factor stimulation; often the basal level of expression is quite low (15,20). Northern blotting of an adult human multiple tissue blot demonstrated a widespread expression of SOCS2 mRNA (Fig. 2A). Interestingly, the strongest SOCS2 mRNA expression was in the heart, placenta, and lung, three highly vascularized organs.

### Novel Association Between SOCS2 and VEGFR1 and VEGFR2

Using an affinity purified rabbit polyclonal antibody against His-SOCS2 to probe lysates from transfected cells, Flag-SOCS2 was readily detected by Western blot (Fig. 2B lane 2). More importantly, SOCS2 protein was expressed in several endothelial cell lines, including PAE, EA, and Py-4-1 (Fig. 2B). Socs2 protein was also readily detected in a term placenta by Western blot. Immunohistochemistry on fresh-frozen human placental tissue demonstrated SOCS2 expression in the endothelium of both large vessels and small vessels perfusing the chorionic villi (Fig. 3 A,D). The demonstration of SOCS2 protein expression in the endothelium supports the functional significance of an interaction between SOCS2 and the VEGF receptors *in vivo*.

### **SOCS2 interaction with VEGFR1 and VEGFR2 requires both receptor kinase activity and an intact SOCS2 SH2 domain**

To further investigate the nature of the association between SOCS2 and the VEGFRs, we used a GST-pulldown assay. In this experiment, wild-type GST-VEGFR1 associated with SOCS2 from transfected 293 cell lysates, confirming the interaction seen in the yeast two-hybrid system (Fig. 4A lane 2 Flag). As in the yeast two hybrid system, SOCS2 also failed to associate with GST-VEGFR1-KR (Fig 4A lane 3 Flag), confirming the dependence of the interaction on VEGFR1 tyrosine phosphorylation. To investigate the requirement for an intact SOCS2 SH2 domain, a SOCS2 mutant in which the arginine in the FLVRES consensus SH2 binding domain was mutated to lysine (Flag-SOCS2-RK) (18,19). The SOCS2-RK protein did not associate with

Novel Association Between SOCS2 and VEGFR1 and VEGFR2 either wild-type or kinase-inactive VEGFR1 (Fig 4A lanes 6, 7 Flag), demonstrating that this interaction is mediated by SH2 domain-phosphotyrosine interaction.

Several groups have demonstrated PLC $\gamma$  association with phosphorylated VEGFR1 (11,24). To determine the effect of SOCS2 overexpression on PLC $\gamma$  association, we re-probed the Western blots for VEGF-R1-associated PLC $\gamma$ . Roughly equal amounts of PLC $\gamma$  associated with GST-VEGFR1, regardless of the presence of overexpressed SOCS2 or SOCS2RK (Fig. 4A lanes 2,6,10 PLC $\gamma$ ).

Because SOCS2 interacted with VEGFR2 as well as with VEGFR1 in the two-hybrid system, we next tested SOCS2-VEGFR2 interaction in a GST-pulldown assay. Wild-type GST-VEGFR2 associated with SOCS2 (Fig 4B lane 2 Flag) in a phosphotyrosine-dependent manner (Fig 4B lane 3 Flag). SOCS2RK did not associate with either wild-type or kinase-inactive GST-VEGFR2 (Fig 4B lanes 6,7 Flag), demonstrating a requirement for the SOCS2 SH2 domain in the interaction between these proteins.

PLC $\gamma$  has also been demonstrated to associate with activated VEGFR2 (25), but overexpression of SOCS2-wt or -KR did not affect the association of PLC $\gamma$  with wild-type VEGFR2 (Fig. 4B lanes 2,6,10 PLC $\gamma$ ). Therefore, the association of both VEGFR1 and VEGFR2 with SOCS2 is dependent upon both the VEGFR tyrosine phosphorylation and the presence of an intact SH2 domain in SOCS2.

## Discussion

Our results demonstrate a novel association between VEGF receptors 1 and 2 and the SH2 domain-containing protein SOCS2. Both VEGFR1 and the closely related VEGFR2 interacted with SOCS2 in a kinase-dependent manner in the two-hybrid system. GST-pulldown experiments confirmed the association between the VEGFRs and SOCS2 and demonstrated that these interactions require an intact SOCS2 SH2 domain. The demonstration of SOCS2 protein expression in cultured endothelial cells and in the endothelium of human tissues *in vivo* further supports the possibility of a biologically relevant interaction between SOCS2 and the VEGFRs.

### SOCS family members negatively regulate cell signaling

The first member of the SOCS family of proteins was discovered with the simultaneous publication of three papers that identified SOCS1 using three different approaches(26-28). SOCS1 is characterized by a central SH2 binding domain, an N terminal region, and a C terminus with a characteristic "SOCS box". Subsequently, other members of the SOCS family were identified by homology to the SOCS box (20,29,30). While the SOCS box can be found C terminal to a variety of domains including ankyrin repeats, Spry domains and WD-40 repeats, SOCS family members all possess a central SH2 domain.

Novel Association Between SOCS2 and VEGFR1 and VEGFR2  
SOCS family members share a common role in the negative regulation of signal transduction

(15). Stimulation of cytokine receptors leads to activation and phosphorylation of Janus kinases (Jaks), which then phosphorylate the STATs. STAT phosphorylation results in homo- or heterodimerization of the STATs, followed by nuclear translocation, and initiation of target gene transcription (31). SOCS proteins are upregulated in response to cytokine activation and then negatively regulate cytokine signaling by disrupting the Jak/STAT pathway, thus completing a classical negative feedback loop.

RTKs can also regulate the Jak/STAT/SOCS pathways. For example, insulin stimulation of 3T3-L1 adipocytes induces SOCS3 expression via STAT5B (32). Once SOCS3 is expressed in the cytosol, it negatively regulates the pathway by competing for STAT5B binding sites on the insulin receptor. Notably, VEGF has recently been shown to activate STAT signaling pathways. VEGF receptor activation has been shown to induce tyrosine phosphorylation, DNA binding activity, and transcriptional activation of STAT3 and STAT5 (33). VEGF stimulation of HUVEC cells induced STAT5 binding activity; STAT5 increased p21 mRNA expression via VEGFR1. VEGF stimulation of BAEC cells resulted in the activation of STAT1 and STAT6 (34). STAT activation by VEGF stimulation suggests that SOCS genes may be upregulated, which, when taken together with our findings, indicates a potential negative feedback regulation of VEGF signaling by SOCS proteins.

#### Novel Association Between SOCS2 and VEGFR1 and VEGFR2

Like other SOCS family members, SOCS2 expression is tightly regulated in many cell types, with low resting levels of expression (15). The nature of SOCS2 induction in the endothelium could be an important determinant of VEGFR-SOCS2 function. A negative feedback model suggests VEGF-induced STAT activation as a candidate for SOCS2 regulation. Other angiogenic growth factors such as the angiopoietins, FGF, or prolactin might also affect SOCS transcription. Similarly, cytokines could impact VEGF signaling by stimulating STAT and upregulating SOCS transcription. The identification of the pathways that regulate SOCS2 protein expression in the endothelium will be important to understand its role in angiogenesis.

#### **Mechanisms of negative regulation by SOCS proteins**

SOCS family members employ several distinct mechanisms for regulating signal transduction. For example, SOCS1 directly binds to and inhibits the JH1 kinase domain of Jak2; its SH2 domain directly binds the critical phosphotyrosine residue in the activation loop of the Jak2 kinase, while the N-terminal kinase inhibitory domain of SOCS1 binds the catalytic groove of the kinase JH1 domain (35). However, CIS and SOCS2 lack the N-terminal kinase inhibitory domain indicating that these members of the SOCS family influence cytokine signaling by an alternative mechanism. Supporting this possibility, preliminary experiments have shown that SOCS2 has no effect on the autophosphorylation of recombinant VEGFR1 (KP and PR unpublished observations).

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SOCS3 can inhibit signaling by either binding Jak2 directly or by competing with other signaling molecules, STAT in particular, for binding sites on activated receptors (36,37). Since SOCS2 lacks an N-terminal kinase inhibitory domain, it may also regulate signaling by competitive binding to phosphorylated sites on the activated receptor (38). The requirement for an intact SH2 domain in SOCS2 for VEGF receptor interaction (Fig 4) suggests that SOCS2 associates with a specific phosphotyrosine residue on the activated VEGF receptor. Since overexpression of SOCS2 did not adversely affect PLC $\gamma$  association (Fig 4), the binding sites of SOCS2 and PLC $\gamma$  on the activated VEGF receptors appear to be different. Future experiments will determine the specific binding site for SOCS2 on VEGFR1 and VEGFR2 and clarify the function of SOCS2 association with the VEGFRs.

SOCS2 can perform different functions depending on its level of cellular expression. Low levels of transfected SOCS2 protein suppress GH induced transcription of STAT5, whereas higher levels actually cause superinduction of transcription (39). SOCS2 has only a partial inhibitory effect on other types of cytokine signaling, regardless of its concentration (15). Coexpression of SOCS2 can modulate SOCS1 suppression of STAT5 transcription at higher concentrations (39,40). Thus, it will be important to determine whether other SOCS proteins are involved in VEGF signaling and how they interact with SOCS2 to modulate angiogenesis.

Novel Association Between SOCS2 and VEGFR1 and VEGFR2

**Function of SOCS2 expression in angiogenesis**

In order to determine the function of SOCS2 in the VEGF-induced angiogenic response, it will be necessary to alter SOCS2 expression in functional assays. While the association of SOCS2 with both VEGFR1 and VEGFR2 suggests that the SOCS protein acts as a general modulator of the VEGF response, stimulation with PlGF vs. VEGF or receptor-specific mutants of VEGF will reveal the specificity of SOCS2 regulation of the VEGF receptors *in vivo*.

The recently published SOCS2 knockout mice had no obvious vascular defects (41). The predominant phenotype –gigantism– appears directly attributable to upregulation of Growth Hormone via an increase in IGF-1 production. Although a vascular phenotype was not reported, it is obvious that a larger vascular bed would be required to support a larger body mass. Overexpression of VEGF results in increased tissue vascularity, thus it is interesting to speculate that lack of a potential negative regulatory protein like SOCS2 might have resulted in increased numbers of blood vessels, a feature that was not specifically evaluated. On the other hand, it is possible that redundancy among the SOCS proteins and/or other negative regulatory mechanisms compensated for the lack of SOCS2 and prevented significant vascular defects. Thus, mice lacking SOCS2 or other SOCS proteins may provide a unique opportunity to study the role of these proteins in modulating VEGF-mediated angiogenesis.

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**Figure Legends**

**Figure 1: Bait testing in the yeast-two hybrid system demonstrated a kinase- dependent association between VEGFR1 and VEGFR2.**

The VEGFR1 cytoplasmic domain was used as bait in a yeast-two hybrid screen of a human fetal heart library (gift of M. Blonar, Bristol-Myers Squibb). This screen demonstrated a novel association between VEGFR1 and SOCS2, an SH2-domain containing cytoplasmic protein. SOCS2 was then tested for association with the wild type and kinase-deficient (KR, with a point mutation in the ATP-binding site) cytoplasmic domains of VEGFR1 and VEGFR2.

**Figure 2: SOCS2 mRNA expression in human tissues and SOCS2 protein expression in placenta and endothelial cells.**

A: SOCS2 mRNA is widely expressed, with highest expression in highly vascular tissues. A human multiple tissue Northern blot was probed with a human SOCS2 probe. H=heart, Br=brain, Pl=placenta, Lu=lung, Li=Liver, SkM=skeletal muscle, K=kidney, Pan=pancreas. B. SOCS2 protein is detectable in endothelial cell lines and human placental lysate. An affinity purified rabbit polyclonal anti-SOCS2 was used to probe lysates from PAE and PAE transfected with Flag-SOCS2 demonstrating antibody recognition of both the endogenous and Flag-tagged

Novel Association Between SOCS2 and VEGFR1 and VEGFR2 versions of SOCS2. Endogenous SOCS2 is also detectable in EA, Py-4-1, and human placenta but not in HUVEC.

**Figure 3: Immunohistochemistry demonstrates SOCS2 protein expression in the endothelium of placental blood vessels.**

Sections of a term human placenta were probed with an affinity purified polyclonal antibody against human SOCS2. SOCS2 protein is present in both the large vessels of the placenta (A) as well as the smaller vessels of the chorionic villi (D) as compared to affinity purified preimmune serum (B, E) and CD31, an endothelial cell marker (C, F).

**Figure 4: GST-VEGFR1 and GST-VEGFR2 kinase associate in a kinase-dependent manner with the SH2 domain of SOCS2.**

Glutathione beads were bound to: C, sf9 lysate alone; wt, wild-type GST-VEGFR2 kinase domain; KR, GST-VEGFR kinase-inactive kinase domain. Beads were incubated with lysates from 293 cells transfected with: pFlagSOCS2 (wild-type Flag-SOCS2), pFlagSOCS2RK (Flag-SOCS2 with a point mutant in the FLVRES consensus sequence for SH2 domain binding); or empty pFlag vector. The pulldowns are first three lanes of each set, alongside a control lane (L), containing 293 lysates. Western blots are for Flag, GST and PY99, and PLC $\gamma$ . A. GST-VEGFR1 kinase associates with the SH2 domains of SOCS2 in a kinase-dependent manner.

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SOCS2 associated with GST-VEGFR1 [lane 2, Flag], association is dependent upon the tyrosine phosphorylation of GST-VEGFR1 [lane 2 vs lane 2, Flag] and the presence of a functional SOCS2 SH2 domain [lane 2 vs lane 6, Flag]. B. SOCS2 association with VEGFR2 is dependent upon both tyrosine phosphorylation of the VEGFR2 kinase and the presence of a functional SOCS2 SH2 domain. SOCS2 association with GST-VEGFR2 [lane 2, Flag] is dependent upon the tyrosine phosphorylation of GST-VEGFR2 [lane 2 vs lane 2, Flag] and the presence of a functional SOCS2 SH2 domain [lane 2 vs lane 6, Flag].

Figure 1

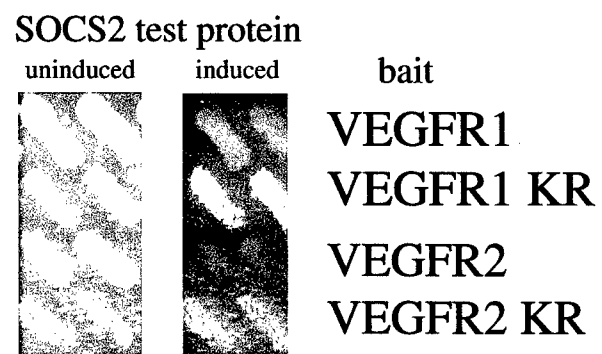
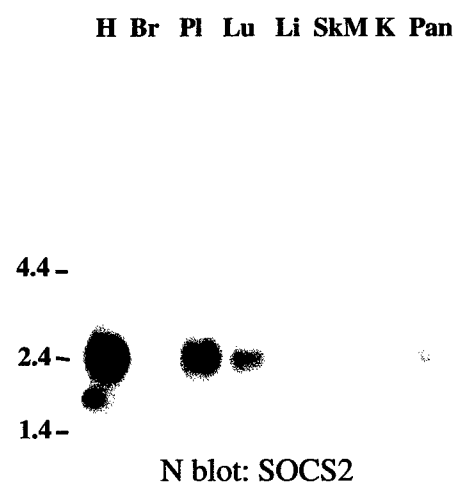


Figure 2

A



B

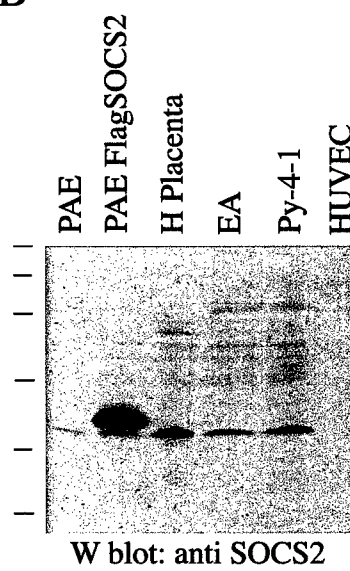
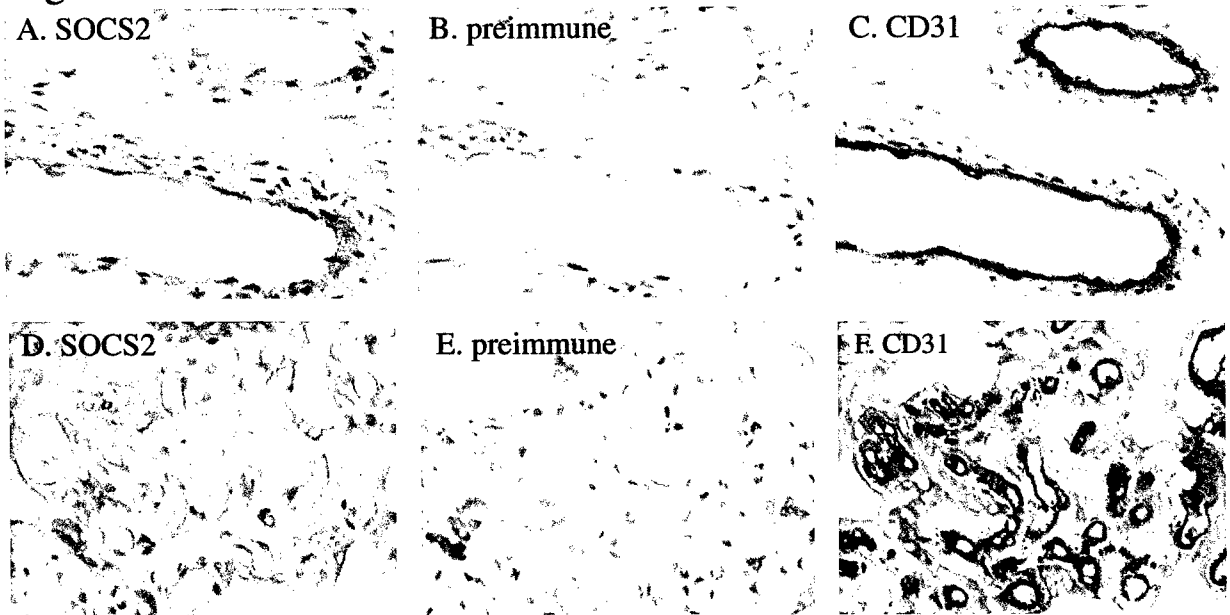
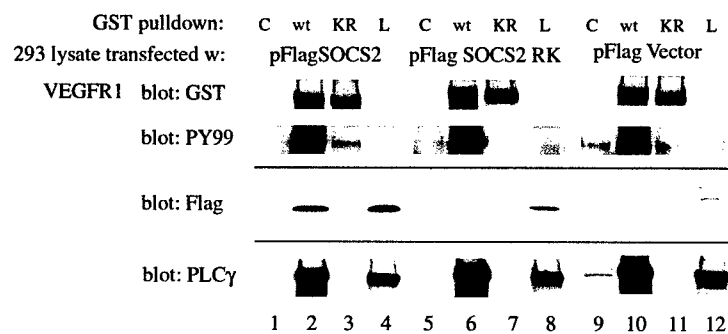


Figure 3

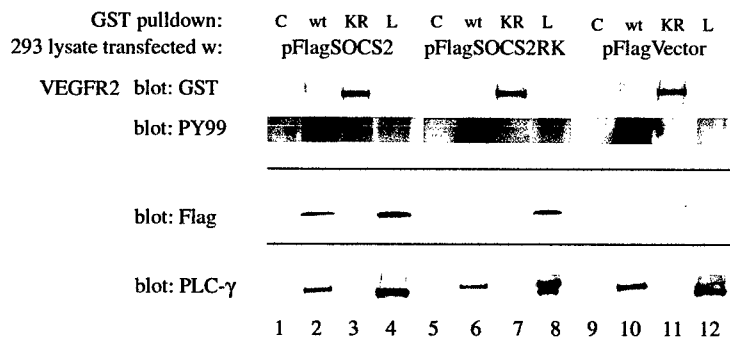


## Figure 4

### A: GST-VEGFR1



### B: GST-VEGFR2





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FORT DETRICK, MARYLAND 21702-5012

REPLY TO  
ATTENTION OF:

MCMR-RMI-S (70-1y)

5 Mar 02

MEMORANDUM FOR Administrator, Defense Technical Information  
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,  
VA 22060-6218

SUBJECT: Request Change in Distribution Statements

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for grants. Request the limited distribution statements for Accession Documents listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART  
Deputy Chief of Staff for  
Information Management

DISTRIBUTION TO BE CHANGED TO UNLIMITED,  
APPROVED FOR PUBLIC RELEASE

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